

ROLES OF OXYGEN AND CARBON DIOXIDE IN THE CONTROL OF SPIRACULAR FUNCTION IN CECROPIA PUPAE

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In most insects spiracular valve movements are controlled by oxygen and carbon dioxide (Beckel and Schneiderman, 1957; Schneiderman, 1960; Levy and Schneiderman, 1966a, 1966b). When the intratracheal concentration of carbon dioxide is high or that of oxygen is low, the spiracular valves open; when the intratracheal concentration of oxygen is high, they close. However, the sites at which these gases act have never been demonstrated. The central nervous system is surely involved (Miller, 1966) as are the spiracles themselves. This report identifies the targets of oxygen and carbon dioxide in diapausing pupae of the *Cecropia* moth, discusses the interaction of oxygen and carbon dioxide in controlling spiracular behavior, and analyzes several aspects of the nervous control of the spiracular closer muscle.

MATERIALS AND METHODS

Diapausing pupae of the silkworm *Hyalophora cecropia* were used. They were stored in their cocoons at 2° C and 90% R.H. until they were prepared for experiments as follows: their abdomens were immobilized with strips of paraffin (Schneiderman and Williams, 1955) to prevent internal movements from damaging the ganglion preparation, and external telescoping movements from obscuring the spiracles of movable segments. The brain was removed to insure permanent diapause (Williams, 1946). Pupae then were allowed to recover for at least two weeks at 25° C and 50% R. H.

Two days before an experiment was begun the pupa was provided with an "inlet" and an "outlet" cannula (Fig. 1) which enabled us to perfuse the entire tracheal system, including the tracheae serving the spiracular muscles ("spiracular" flow). Cannulae were 8 cm lengths of polyethylene tubing (0.97 o.d., 0.58 mm i.d. (PE-50); or 0.61 mm o.d., 0.28 mm i.d. (PE-10)) (Intramedic tubing, Clay-Adams Company, New York). Each cannula was inserted past the spiracular valve (Schneiderman and Schechter, 1966; Brockway and Schneiderman, 1967). The cannulae were checked for leaks as described by Brockway and Schneiderman (1967). The experimental gas was introduced into the tracheal system via the second or third right abdominal spiracle, and excess gas escaped via either the fifth or sixth left abdominal spiracle.

The fourth abdominal ganglion was selected for neural and tracheal surgery since it is located conveniently in a non-collapsible segment, the spiracles of which are not obscured by overlying pupal wingpads. The spiracle on the right side of that segment was exposed and observed in all experiments. In addition

the third or fifth right abdominal spiracle or both were exposed and observed as control spiracles.

The condition of the exposed spiracular valves was checked daily by probing the pupa gently on the cuticle in the area of the exposed spiracles; this maneuver caused properly functioning valves to open. In air, valves of a normal pupa pulsate most of the time, that is, they move but do not open. At the beginning of each experiment moist air was perfused through the pupa's tracheal system; if the valves failed to pulsate, the pupa was discarded.

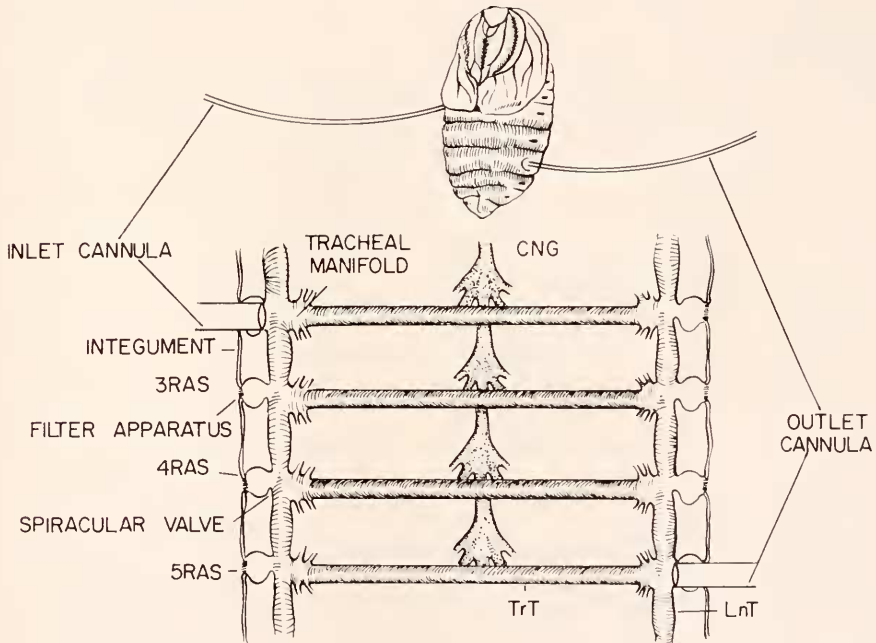


FIGURE 1. Arrangement of cannulae for spiracular flow. The upper part of the diagram shows a pupa with the inlet and the outlet cannula in position; the lower part of the figure shows a portion of the tracheal system perfused via this arrangement. The inlet cannula (2RAS, above 3RAS) was connected to the perfusion pump, and the outlet cannula (5LAS, opposite 5RAS) was open to the atmosphere. Gas entering the inlet cannula flowed throughout the tracheal system; excess gas escaped via the outlet cannula; 3RAS, 4RAS, 5RAS—third, fourth, fifth right abdominal spiracle; CNG—ganglion; TrT—transverse trachea; LnT—longitudinal trachea.

Animals were mounted ventral side up in modeling clay for observations. Experiments were performed at 22–25° C and 40–50% R.H. During the recovery intervals between gas perfusions the pupa was kept in a water-saturated environment.

Anatomy of the spiracle

The spiracular apparatus was described in detail by Beckel (1958) (Fig. 2). It consists of a membranous spiracular valve, a chitinous frame which acts as a

lever, and the spiracular closer muscle which is opposed by an elastic ligament and is innervated by a single spiracular nerve (Fig. 3).

Recording spiracular valve movements

The method described by Schneiderman (1956, 1960), in which the leading edge of the spiracular valve is followed in an ocular micrometer, was used for recording valve movements. A Grass FT 03 force displacement transducer and polygraph (Grass Instrument Company, Quincy, Massachusetts) were substituted for the lever-pen system. All except the most rapid and frequent valve movements could be followed and recorded almost as soon as they occurred. It was possible to record the movements of only one valve at a time. However, all spiracular valves engage in the same mode of behavior even though they are not synchronous (Van der Kloot, 1963; Brockway and Schneiderman, 1967), *i.e.*,

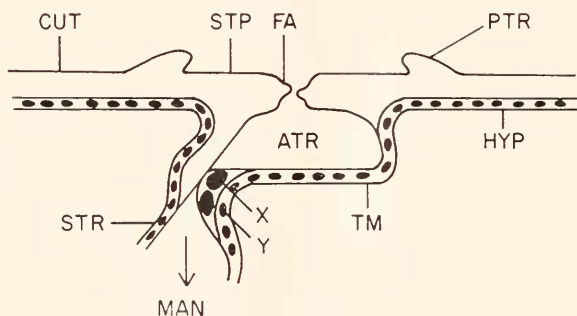


FIGURE 2. Frontal section through the spiracular region (redrawn from Beckel, 1958). The closing bars push the tracheal membrane against the closing bow, thus closing the spiracular opening as in this drawing; ATR—atrium; CUT—cuticle; FA—filter apparatus; HYP—hypodermis; MAN—manifold; PTR—peritreme; STP—stigmal plate; STR—spiracular trachea; X, Y—dorsal lateral and dorsal median closing bar, respectively.

they constrict, flutter, or open fully. Hence the behavior of the observed valve is qualitatively like that of the others. A dissecting microscope permitted viewing of several valves in turn at $30\times$ without disturbing the preparation. Consecutive recordings lasting 10 seconds to 3 minutes each were made when observations of two or more valves were required.

Gas perfusions

Gas mixtures were made up in small cyclinders or directly in a 50 ml syringe from commercially available cylinders of oxygen, carbon dioxide, and nitrogen. The syringe was equipped with a three-way Luer-Lok MS 02 valve which permitted direct filling of the syringe from cylinder lines at approximately atmospheric pressure, and minimized the possibility of contaminating the contents of the syringe with external air. The syringe could be refilled in less than one minute when long perfusions were necessary. Gas analyses accurate to $\pm 1\%$ were performed, using a Model E2 paramagnetic oxygen analyzer and a Model 215 infrared carbon

dioxide analyzer (Beckman Instruments, Inc., Fullerton, California). As recommended by the manufacturer, neoprene tubing was used for cylinder lines and Viton-A vacuum tubing (Bearings, Inc., Cleveland, Ohio) connections were used with the analyzers. These materials are impermeable to the gases used in these experiments. A hypodermic needle was attached to the syringe valve; a 50 cm length of polyethylene tubing the same diameter as the inlet cannula of the pupa connected the needle with the cannula. The plunger of the syringe was driven at a constant predetermined rate by a motor. Two syringes were used when two gas mixtures were perfused simultaneously.

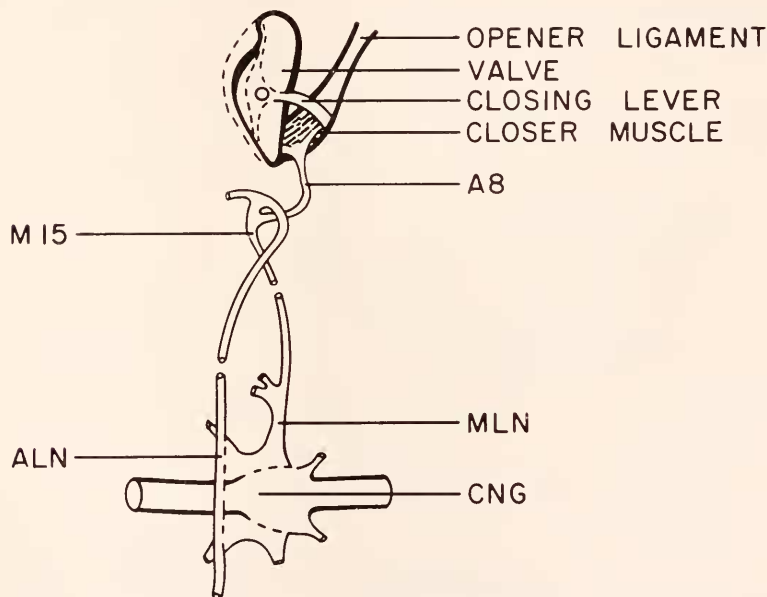


FIGURE 3. Innervation of an abdominal spiracular closer muscle (redrawn from Beckel, 1958). CNG—ganglion; ALN—anterior lateral nerve; MLN—mid-lateral nerve; A8—branch of ALN innervating the muscle; M15—branch of MLN innervating the muscle.

Animals were perfused at a rate of $360 \mu\text{l}/\text{min}$ (occasionally $1430 \mu\text{l}/\text{min}$) for at least 20 minutes or until a definite unchanging pattern of spiracular response had been established. A 20-minute recovery period was allowed between exposures to different gases although normal behavior of spiracular valves, assuming that behavior in ambient air is "normal," was resumed usually within 10 minutes after the gas flow had been terminated.

As a routine check on the initial condition of the pupa, and to determine the pattern of spiracular valve activity, the following procedure was used: The tracheal system of each of 59 pupae was perfused before the experiment began with at least 5 of the following: 2, 3, 5, 10, 21 (air), or 50% O_2 (balance N_2 and CO_2). Since the tracheal system normally contains at least 3% CO_2 (Levy and Schneiderman, 1966b), 3 or 4% CO_2 was added. Similar results were obtained whether the concentration of oxygen was selected at random or progressively increased

from 2 or 3% to 21 or 50%. Records of valve responses by each pupa to different gas mixtures were used as controls for subsequent experimental records of that same pupa.

Perfusing the tracheae of an abdominal ganglion

To provide the fourth abdominal ganglion with its own gas supply, separate from that of the rest of the pupa, the following procedures were used:

An 8 mm square of integument overlying portions of the third, fourth, and fifth abdominal segments was removed. A few crystals of a 1:1:1 mixture

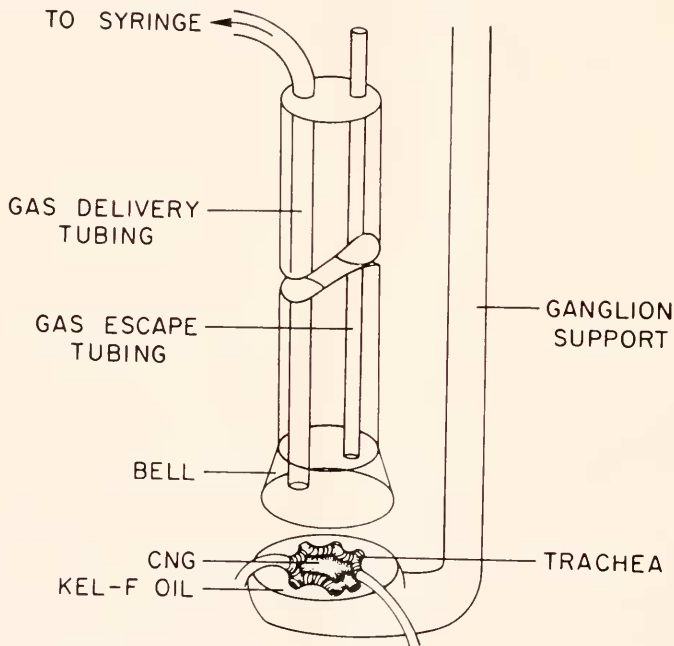


FIGURE 4. Assembly for supporting the fourth abdominal ganglion and perfusing it with the ganglionic flow. The bowl of the ladle was filled with Kel-F oil to retard desiccation and to prevent the ganglion from sticking. The gas escape tubing permitted excess gas to escape and prevented the Kel-F oil from bubbling; CNG—ganglion.

of phenylthiourea, streptomycin sulfate, and penicillin were placed in the wound. Any lost hemolymph was replaced with insect Ringer (Ephrussi and Beadle, 1936). The same gas mixtures that were used before surgery were perfused through the pupa's tracheal system immediately after surgery. Thus the spiracular valve responses of a pupa to various oxygen concentrations just before and just after injury could be compared.

Following this second set of perfusions, all fat body was removed from the vicinity of the third and fourth abdominal ganglia. This procedure did not interrupt the tracheal supply to the spiracular closer muscle of the fourth spiracle or that of its adjacent control spiracles. The intact fourth abdominal ganglion was

supported from its dorsal side in the bowl of a "ladle" which was inserted beneath the ganglion with a micromanipulator (Fig. 4). A second micromanipulator positioned the gas delivery bell directly over the ganglion. The bell completely enclosed the ganglion resting on its support but did not compress the nerves or tracheae supplying the ganglion. The entire assembly dipped into a drop of Kel-F high polymer oil (Minnesota Mining and Manufacturing Company, St. Paul, Minnesota) so that no external gases contaminated the experimental gas flow. Thus the fourth abdominal ganglion could receive its own separate, localized

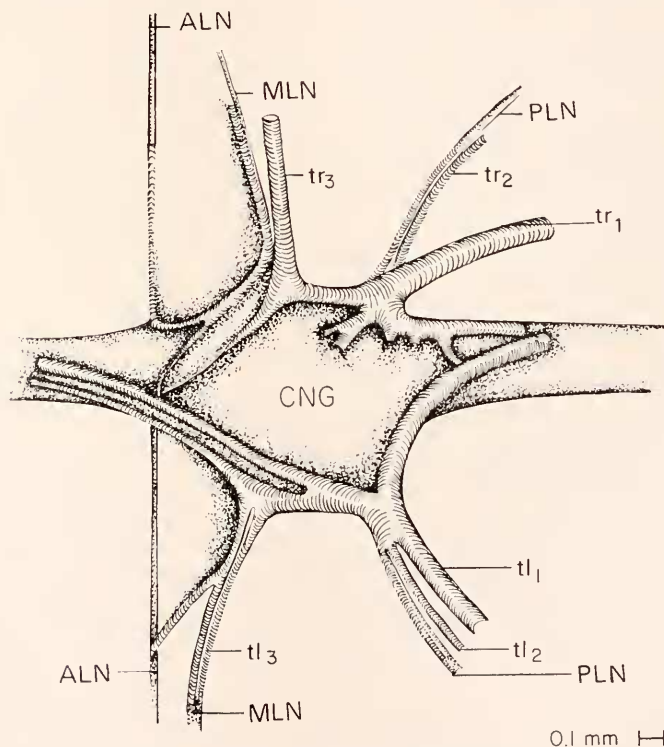


FIGURE 5. Gross anatomy of the immediate tracheal supply to the fourth abdominal ganglion; ALN—anterior lateral nerve; MLN—mid-lateral nerve; PLN—posterior lateral nerve; CNG—ganglion; tr_{1-3} , tl_{1-3} —tracheae.

gas flow via this arrangement ("ganglionic" flow) while a second gas could be perfused simultaneously through the animal's tracheal system (spiracular flow). The third abdominal ganglion served as a control.

At this point, the *intact* fourth abdominal ganglion of each of six pupae was perfused with its separate ganglionic flow, while the spiracles were perfused simultaneously with the spiracular flow, which consisted of the same or a different gas mixture. The perfusions were begun within a minute after the ganglion had been exposed and supported. The effects on spiracular valve behavior of surgery itself and of gas flow over the surface of the intact ganglion could be compared.

After these perfusions the delivery bell was raised, and tracheae tr_1 , tr_2 , tr_3 , tl_1 , tl_2 , and tl_3 (Fig. 5) were cut at their junction with the ganglion; thus the ganglion was "detracheated." This permitted gases flowing over the ganglion to enter the open ends of tracheae attached to it, and eventually to reach the cells in the ganglion. In addition those tracheal branches passing along and attached to the ventral nerve cord near the ganglion were punctured with a fine glass needle to avoid damaging the nerve cord, and then the tracheal ends were teased apart gently. To prevent fluid from creeping into the cut ends of the tracheae attached to the ganglion, thus blocking the ganglion from the experimental gas flow, the tracheae were not severed until just before the bell was lowered over the ganglion and the gas perfusions were begun. During intervals between experiments, air was perfused over the ganglion to prevent hypoxia.

To assess the long-term effects of detracheation, a plastic window could be sealed over the wound in the integument with melted paraffin. Pupae then were stored at room temperature as described above, and examined periodically.

Each experiment reported in this paper was performed on at least two and as many as eight pupae.

Departures from these general procedures as well as special methods will be explained in the appropriate sections below.

RESULTS

Effects of routine experimental procedures on spiracular valve behavior

Four major procedures—cannulation, use of spiracular windows, tracheal perfusion, and surgery—were followed routinely to prepare pupae for experiments. To evaluate the effects of each procedure, the normal behavior of the spiracular valves was used as the standard. Since valves of a pupa respiring in ambient air pulsate most of the time and occasionally flutter (Schneiderman, 1960), this pattern was chosen as "normal."

Cannulation. Four different diameters of polyethylene tubing were tested as cannulae. Spiracular valve responses to air perfusions through cannulae of different bores were the same. PE-10 tubing generally was used because it was the most convenient.

Five pupae were cannulated, each with a different combination of spiracles as cannulation sites. Spiracular valves of these pupae responded in the same way to air perfusions. Thus cannulation itself, the cannula bore size, and the sites of cannulation did not affect the behavior of spiracular valves.

Spiracular window. A plastic window was sealed over exposed valves to prevent the pupa from desiccating. The behavior of the spiracular valves of seven intubated pupae, first with and then without the window, was the same in response to air perfusions. Thus spiracular valve behavior was not visibly affected by the presence of a plastic window over the exposed spiracles.

Tracheal perfusion. The object of perfusing the tracheal system was to control the intratracheal gas composition. To meet this objective we considered the structure of the tracheal system and the rate at which gases were perfused through it. In addition we determined the time needed for equilibration of intratracheal gas with the perfused gas, the time needed for recovery from a perfusion, and whether valve responses to a given gas mixture are reproducible.

Gross dissection of a pupa reveals that tracheae form an interconnecting network. Large tracheae run longitudinally between spiracular manifolds from which numerous smaller transverse tracheae branch and rebranch repeatedly. There are no valves to cause unidirectional flow within pupal tracheae (Brockway and Schneiderman, 1967). Furthermore, when air is perfused through the tracheal system at a high rate, the pupa distends.

The flow rates used in these experiments were a compromise between rates high enough to insure constant average intratracheal levels of oxygen and carbon dioxide and rates low enough to prevent the pupa from distending. It was determined that a flow rate of 360 $\mu\text{l}/\text{min}$ does not itself visibly affect valve behavior yet still meets the oxygen requirements of a pupa, whereas flow rates greater than 1430 $\mu\text{l}/\text{min}$ cause the pupa to distend.

After 2 to 5 minutes of perfusion, valve behavior stabilized and usually remained the same until perfusion ended, usually 20 minutes but occasionally up to 4 hours later. In only eight of over 600 perfusions did valve behavior after 20 minutes differ from that after 2 to 5 minutes of perfusion. Moreover, results were highly reproducible from day to day in any individual and among individuals.

The resumption of normal valve behavior after perfusion had ended was interpreted as recovery and usually occurred within 5 to 10 minutes after perfusion ceases. The duration of perfusion had no visible effect on recovery time.

Similar valve responses to a given oxygen concentration occurred not only in the different spiracles of the same pupa but also among different pupae. Deviations were in the degree of response, not in different modes of behavior. For example, valves of 50 pupae fluttered at amplitudes ranging from low to high in response to perfusions of 15% O_2 ; there were no prolonged valve openings or valve constrictions in any pupa.

Surgery. Injury increases the metabolism of brainless diapausing pupae severalfold (Schneiderman and Williams, 1955). This increased metabolism is conspicuous about 24 hours after injury, becomes maximal about 4 days after injury, and persists from one to several weeks. It was important to know what effect surgery *per se* had on spiracular valve behavior, aside from any specific effect resulting from tracheal or neural surgery.

Each of 14 pupae that had cyclical respiratory activity before integumentary surgery was examined immediately after surgery for burst cycles. Only one pupa continued to exhibit cyclical respiratory activity after injury; its cycles were about 50 minutes long after surgery as compared to about 80 minutes before surgery. Valves of the 13 other pupae fluttered at low amplitude immediately after injury, although several minutes of constriction with pulsations were common.

The spiracular valve responses of injured pupae to different perfused gas mixtures were also examined, and compared to the responses of the same pupae to the same gas mixtures before injury. The valve responses of an individual pupa to a given perfused gas mixture were generally the same after injury as before. In five pupae, post-injury valve responses were recorded almost 72 hours after surgery, at a time when injury metabolism was near maximum. Changes in valve behavior after injury were limited to an increase in flutter amplitude, or to motionless, fully-open valves whereas before injury the fully-open valves occasionally closed partially.

Spiracular valve responses of intact intubated pupae to gas perfusions

It was necessary to know how the spiracular valves of intubated pupae that had not undergone surgery responded to various mixtures of carbon dioxide and oxygen. Thus pupae were perfused with gas mixtures in which the concentration of oxygen varied between 2 and 50%, and that of carbon dioxide ranged from 0 to 7%. Records of valve behavior under these conditions were obtained for

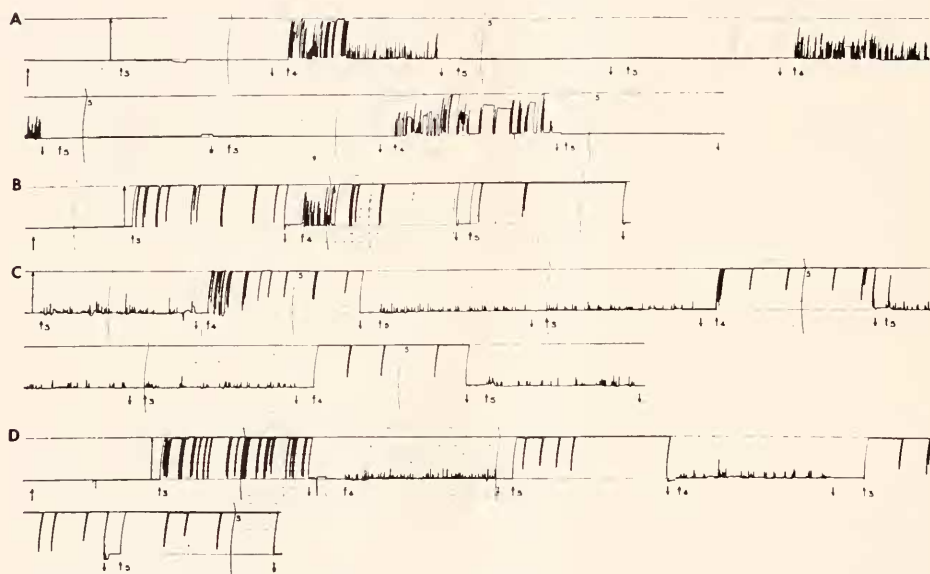


FIGURE 6. Spiracular valve responses to simultaneous spiracular and ganglionic perfusions following detractionation of the fourth abdominal ganglion. The large arrow at the beginning of each record indicates the amount that the pen was deflected when valves opened fully. The short arrows below the tracings indicate the beginning (upward-pointing arrows) and end (down-pointing arrows) of recording. The short breaks in recording represent the time required to bring the next valve in focus (chart speed = 0.5 mm/sec; each division on the chart represents 50 seconds); 3RAS, 4RAS, 5RAS—third, fourth, fifth right abdominal spiracle, respectively; (A.) Spiracular flow—50% O_2 + 3% CO_2 ; ganglionic flow—3% O_2 + 3% CO_2 ; (B.) Spiracular flow—3% O_2 + 3% CO_2 ; ganglionic flow—3% O_2 + 3% CO_2 ; (C.) Spiracular flow—21% O_2 + 3% CO_2 ; ganglionic flow—3% O_2 + 3% CO_2 ; (D.) Spiracular flow—3% O_2 + 3% CO_2 ; ganglionic flow—21% O_2 + 3% CO_2 .

each pupa, and served as a type of control record for subsequent experimental records for that same pupa. The following results were obtained:

In 61 of 101 perfusions of 2 to 5% O_2 and 0 to 4% CO_2 , valves opened; in the 50 other perfusions, valves fluttered at high amplitude. Since the concentration of carbon dioxide in all perfusions was too low to cause opening (Levy and Schneiderman, 1966a), the wide opening of the valves was a response to low oxygen.

When the concentration of carbon dioxide was 0 to 4%, while that of oxygen was 5 to 15%, valves fluttered in 143 of 151 perfusions.

When the concentration of carbon dioxide was 3% and oxygen was 50%, valves were constricted and motionless.

When the concentration of oxygen was too high to cause prolonged valve opening, *i.e.*, above 5%, valves could be induced to remain open by perfusion mixtures containing 7% or more carbon dioxide.

TABLE I
Effect of carbon dioxide concentrations on the valve behavior of the fourth right abdominal spiracle

Pupa no.	Gas mixture				Valve response* 4RAS**
	Spiracular		Ganglionic		
	% O ₂	% CO ₂	% O ₂	% CO ₂	
10-1			3	3	5
			5	3	2
10-2			5	7	1
			5	7	1
			8	12	1
			8	15	1
			8	20	1
			8	25	1
			8	30	1
			8	32	4
			8	35	6
10-1			8	40	6
	21	3	21	3	1
	21	3	5	7	1
	5	7	5	7	5
	5	7	21	3	5
	50	3	50	3	0
	50	3	5	7	0
	5	7	5	7	6
	5	7	50	3	6
	10-2	8	3	8	3
8		3	8	12	1
8		12	8	12	5
8		12	8	3	5

* 1 = closed, motionless; 2 = closed, pulsating; 3 = low amplitude flutter; 4 = medium amplitude flutter; 5 = fully open, moving; 6 = fully open, motionless.

** 4RAS = fourth right abdominal spiracle.

In summary, intratracheal carbon dioxide concentrations of 7% or more were required to cause valves to open fully and remain open when the intratracheal oxygen concentration was 5 to 21%. When the carbon dioxide concentrations were too low to cause valve opening, the valves behaved as follows: they opened fully or fluttered at high amplitude in 2 to 5% oxygen; fluttered at low amplitude in 5 to 15% oxygen; constricted in 21% oxygen or higher. These results agree well with those obtained earlier by other methods (Schneiderman, 1960; Levy and Schneiderman, 1966a, 1966b).

TABLE II

Effect of oxygen concentrations on the valve behavior of the fourth right abdominal spiracle

Pupa no.	Gas mixture				Valve response* 4RAS**
	Spiracular		Ganglionic		
	% O ₂	% CO ₂	% O ₂	% CO ₂	
10-1	3	3	3	3	5
	3	3	21	3	1
	21	3	21	3	1
	21	3	3	3	3
	5	3	5	3	2
	5	3	21	3	1
	21	3	21	3	1
	21	3	5	3	2
10-2	3	3	3	3	5
	3	3	21	3	1
	21	3	21	3	1
	21	3	3	3	5

* 1 = closed, motionless; 2 = closed, pulsating; 3 = low amplitude flutter; 4 = medium amplitude flutter; 5 = fully open, moving; 6 = fully open, motionless.

** 4RAS = fourth right abdominal spiracle.

Control of spiracular valve behavior

To determine whether the central nervous system responds to intratracheal oxygen or carbon dioxide, or to oxygen or carbon dioxide dissolved in the body fluids, the exposed fourth abdominal ganglion (with its tracheae intact) was perfused about 6 hours after the square of integument had been removed. When both the ganglion and the tracheal system were perfused simultaneously with the same gas mixture, spiracles three, four, and five responded similarly. Moreover, all valves behaved alike even when the ganglion and the tracheal system were

TABLE III

Effect of oxygen concentration on the CO₂-trigger threshold for spiracular valve opening

Pupa no.	Gas mixture				Valve response*		
	Spiracular		Ganglionic				
	% O ₂	% CO ₂	% O ₂	% CO ₂	4RAS	3RAS	5RAS**
10-1	8	7	21	3	1	3	3
	5	7	21	3	5	5	5
	5	7	50	3	6	6	6
10-2	5	7	21	3	6	6	6

* 1 = closed, motionless; 2 = closed, pulsating; 3 = low amplitude flutter; 4 = medium amplitude flutter; 5 = fully open, moving; 6 = fully open, motionless.

** 3RAS = third right abdominal spiracle; 4RAS = fourth right abdominal spiracle; 5RAS = fifth right abdominal spiracle.

perfused simultaneously, each with a different gas mixture. Apparently tracheae are not very permeable to externally applied gases. It is the tracheal gas *per se* that affects the ganglion, not gases dissolved in the hemolymph (or Kel-F oil) surrounding the ganglion.

The key steps in these experiments were detracheating and perfusing the ganglion with ganglionic flows. Tables I–III include representative data taken from recordings of valve responses to various perfusions following detracheation. Figure 6 shows sample records. Table I shows the effects of carbon dioxide in the ganglionic flow on the valve behavior of the fourth spiracle. When the carbon dioxide concentration was 7 to 30%—concentrations that have been demonstrated repeatedly to cause valve opening when the spiracles are perfused with them—the spiracle remained constricted and pulsating. The carbon dioxide concentration over the ganglion had to be above 30% (pupa 10–2) before the fourth spiracle could be induced to open. Physiological concentrations of carbon dioxide (3 to 7%) perfused through the ganglion had no effect on spiracular behavior. When the spiracles were perfused with 5% O₂ + 7% CO₂, all the spiracular valves opened fully although the ganglionic flow was 50% O₂ + 3% CO₂. On the other hand, 5% O₂ + 7% CO₂ perfused over the ganglion did not cause valve opening of the fourth spiracle. Similarly, a ganglionic flow of 8% O₂ + 12% CO₂ failed to evoke valve opening by the fourth spiracle, although all valves opened fully when the spiracles were perfused with the same mixture.

These data show that the behavior of the spiracles is not affected when the carbon dioxide concentration of the ganglionic flow is in the physiological range, *i.e.*, there is no central carbon dioxide effect. The primary target of carbon dioxide is outside the central nervous system, *i.e.*, peripheral.

The results of 30 of 33 simultaneous spiracular and ganglionic perfusions indicate that the ganglion responded to its own concentration of oxygen, not to that of the spiracular flow (Table II). When 21% O₂ + 3% CO₂ was perfused through the ganglion, the valve of the fourth spiracle closed although the spiracular flow contained 3% O₂ + 3% CO₂ (pupa 10–2). The valve of the fourth spiracle of pupa 10–1 could be induced to flutter by a ganglionic flow of 5% O₂ + 3% CO₂; the valve could be closed again by a ganglionic flow of 21% O₂ + 3% CO₂.

These data show that the ganglion responds to changes in the oxygen concentration of its own gas supply, *i.e.*, there is a central oxygen effect. The data also indicate that each ganglion serves its own spiracles.

Table III shows the effect of the concentration of oxygen in the ganglionic flow on the CO₂-trigger threshold for spiracular valve opening. As shown earlier, a spiracular flow of 5% O₂ + 7% CO₂ causes full opening of all valves. The fourth spiracular valve of pupa 10–1 continued to open fully in 5% O₂ + 7% CO₂ even when the concentration of oxygen in the ganglionic flow was increased to 50%. The few available data indicate that the concentration of oxygen in the ganglionic flow apparently has no effect on the CO₂-trigger threshold for valve opening.

Table III also shows one example of the effect of the spiracular oxygen concentration on the CO₂-trigger threshold for spiracular valve opening. When carbon dioxide was kept constant at 7% and the spiracular oxygen level in-

creased from 5 to 8%, valve responses of the control spiracles changed from fully open in 5% O₂ to flutter in 8% O₂. The fourth spiracular valve, which had been fully open in 5% O₂ + 7% CO₂, constricted and pulsated when the spiracular flow was 8% O₂ + 7% CO₂, and thus responded differently from its control spiracles.

This experiment demonstrated that the spiracular, not the ganglionic, oxygen concentration affects the CO₂-trigger threshold for valve opening. It also provided additional evidence that each ganglion serves its own spiracles.

However, further experiments were performed to locate the primary site of nervous control of spiracular activity. The ventral nerve cord was cut either posterior to, anterior to, or posterior and anterior to the fourth abdominal ganglion,

TABLE IV
Effect of nerve cord transection on valve behavior

Pupa no.	Position of cut	Gas mixture				Valve response*					
		Spiracular		Ganglionic		Before transection			After transection		
		%O ₂	%CO ₂	%O ₂	%CO ₂	4RAS	3RAS	5RAS	4RAS	3RAS	5RAS**
7-1	Anterior to 4AG	10	3	21	0	2	4	4	6	3	3
		21	0	10	3	4	2	2	2	2	2
7-2	Anterior to 4AG	10	3	21	0	2	4	4	2	2	2
		21	0	10	3	3	2	2	4	2	2
7-3	Posterior to 4AG	10	3	21	0	2	4	4	2	4	4
		21	0	10	3	2	4	4	4	2	2
7-4	Anterior and posterior to 4AG	10	3	21	0	2	4	4	2	4	4
10-2	Anterior to 4AG	5	3	3	3	5	2	2	0	0	0
		3	3	50	3	6	6	6	4	5	5

* 1 = closed, motionless; 2 = closed, pulsating; 3 = low amplitude flutter; 4 = medium amplitude flutter; 5 = fully open, moving; 6 = fully open, motionless.

** 3RAS = third right abdominal spiracle; 4RAS = fourth right abdominal spiracle; 5RAS = fifth right abdominal spiracle.

thus isolating the ganglion from nerve impulses arising in ganglia either posterior and/or anterior to it. Table IV contains data derived from recordings of valve responses of pupae that had undergone nerve cord transection. When 50% O₂ + 3% CO₂ was perfused through the ganglion, the fourth spiracle fluttered (pupa 10-2); its controls oscillated about the fully open position in response to a spiracular perfusion of 3% O₂ + 3% CO₂. In most perfusions the behavior of the fourth spiracle differed from that of its controls. Cutting the nerve cord and isolating the fourth abdominal ganglion did not appear to affect the behavior of the fourth spiracle which responded to the ganglionic flow. These results confirm other evidence that a spiracle is served primarily by its own ganglion, not by the ganglion in an adjacent segment.

To further elucidate the role of the nervous system in controlling spiracular behavior, the spiracular closer muscle was partially denervated by cutting the

mid-lateral nerve (Fig. 3) where it leaves the ganglion. The integumentary wound was sealed with a plastic window. Spiracular gas perfusions and recordings of the valve behavior of the fourth right abdominal spiracle and its contralateral and adjacent control spiracles were begun about 9 minutes later. Perfusion and records of valve behavior were made at various times up to 30 days after nerve transection. The partially denervated spiracle remained open for 24 hours even in 50% O₂, while its controls fluttered in 21% O₂ and closed in 50% O₂. Beginning at about 72 hours and continuing as long as 20 days after denervation, the fourth spiracle closed in response to spiracular perfusions of 21 and 50% O₂, but opened fully in 10% O₂. Thereafter the fourth spiracle remained closed but could be induced to open by perfusions of 100% CO₂ for about one minute; the controls continued to flutter in 21 and 10% O₂, and to constrict in 50% O₂.

These results show that the partially denervated spiracular muscle, like the completely denervated spiracular muscle, is capable of responding to carbon dioxide and oxygen (Beckel and Schneiderman, 1957). However, partial denervation alters the degree of the muscle's responses to changes in the oxygen concentration. In addition the muscle's hypersensitivity to a concentration of oxygen that caused its normal controls to flutter was demonstrated.

DISCUSSION

Several procedures used in these investigations are either described for the first time or are modifications of techniques reported in other papers. Since interpreting the results depends on understanding the advantages and limitations of each method used, two key experimental approaches, intratracheal perfusion and detraction of the ganglion, are discussed before the results are interpreted.

It is important not only to know the tracheal gas composition but also to be able to control it. One way of doing this is opening the pupal tracheal system to ambient gases of known composition by inserting short tubes past the spiracular valves (Buck and Keister, 1955; Schneiderman, 1960). This approach has the advantage of not affecting the animal by mass internal flows. The tracheal gas composition is not known with certainty "but at least it can be kept constant and the oxygen and carbon dioxide tensions can be varied independently" (Schneiderman, 1960, page 520). The behavior of spiracles when the pupal tracheal system was perfused with various gases was very similar to the behavior of spiracles when the insect was placed in the same gases. However, intratracheal perfusion makes it possible to control the average tracheal gas composition.

Detraction of a ganglion cuts its normal oxygen supply route, thus making the ganglion hypoxic. One would expect the valves of the same segment to gape as they do initially after exposing a denervated spiracular muscle to low oxygen (Beckel and Schneiderman, 1957), and this happened. Detraction of a ganglion produces "functional denervation" of the pair of spiracular muscles it serves. The fact that the detracted ganglion perfused with appropriate gas mixtures did not cause the spiracles to gape is evidence that the method of perfusing the ganglion worked.

The interaction between carbon dioxide and oxygen in triggering bursts in diapausing pupae of Lepidoptera has been described (Schneiderman, 1960; Levy

and Schneiderman, 1966b). Their results, based both on observations of spiracular valve behavior and on analyses of tracheal gases, showed that the triggering carbon dioxide concentration varied with the tracheal oxygen concentration. The carbon dioxide concentration needed to cause prolonged valve opening increased as the intratracheal oxygen concentration increased. Results of the present experiments confirm these earlier reports. Moreover, our data also illuminate another aspect of oxygen-carbon dioxide interaction, the effect of the carbon dioxide concentra-

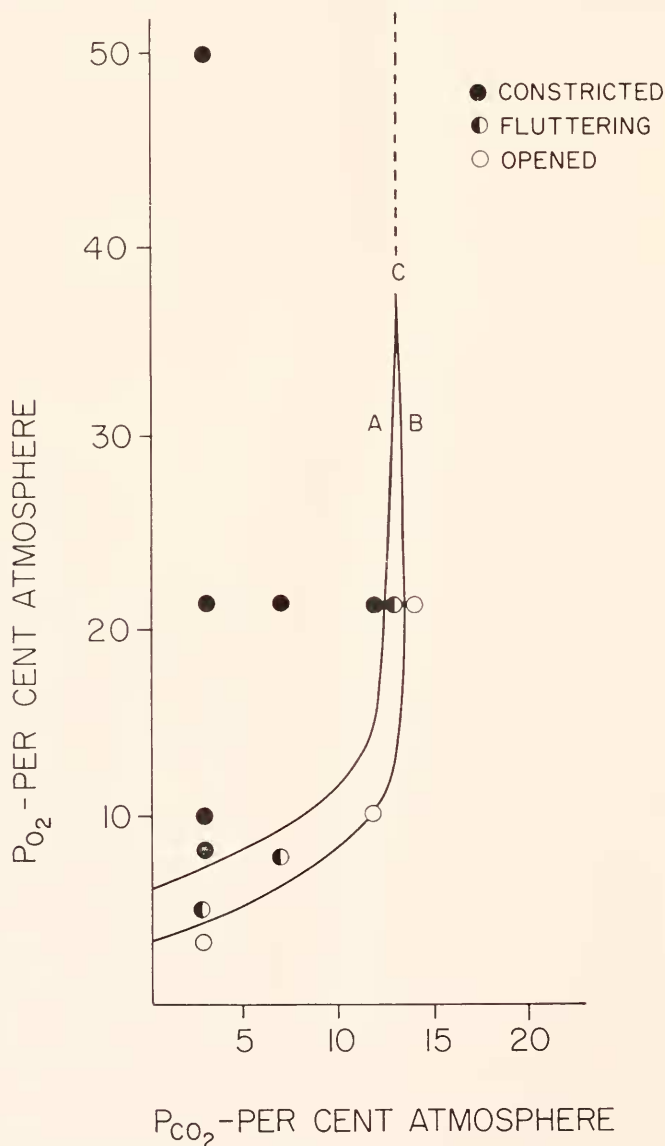


FIGURE 7. Oxygen-carbon dioxide interaction curves for pupa 10-2. See text for details.

tion on the O_2 -flutter threshold of the valves (the concentrations of oxygen at which fluttering begins).

If one plots the intratracheal oxygen-carbon dioxide concentrations at which flutter occurs, in addition to the concentrations of these gases that cause valve opening, the two resulting curves define valve behavior over a range of oxygen and carbon dioxide concentrations. Using data obtained from perfusion experiments, such dual O_2 - CO_2 interaction curves were constructed (Fig. 7). The area between curves A and B includes all combinations at which flutter was observed; the area between the ordinate and curve A includes all combinations at which pulsation or constriction occurred. The area delimited by curve B, the ordinate, and the abscissa includes all combinations for which valves opened. These curves show that when the level of carbon dioxide is sufficiently high, flutter occurs at oxygen concentrations to which valves usually respond by constricting. Thus flutter occurred in 21% O_2 when the carbon dioxide concentration was about 14%; the valves of this same pupa constricted and pulsed in 21% O_2 when the carbon dioxide concentration was 3%. Levy and Schneiderman (1966a) acknowledged the possibility that carbon dioxide could influence the O_2 -flutter threshold but they stated that "until P_{CO_2} rises above 6% it exerts no marked influence on the response of the spiracles to P_{O_2} " (page 97). Results of the present experiment (In the normal respiratory activity of diapausing *Cecropia* pupae, flutter can and whereas marked effects are evident at carbon dioxide concentrations above 10%, results confirm their conclusion, and reveal no conspicuous effects of carbon dioxide on the O_2 -flutter threshold when the carbon dioxide concentration is below 7%, does occur when the intratracheal oxygen concentration is between 18 and 21%. This phenomenon will be discussed in a subsequent paper (Burkett and Schneiderman, 1974).)

Further evidence that increasing the concentration of carbon dioxide affects the flutter threshold comes from re-examining data derived from tracheal gas analyses (Levy and Schneiderman, 1966b, pages 108, 109, Figures 2a, 3a). If these data are equated to corresponding valve behavior, they can be plotted to show the same relationships as in Figure 7, confirming qualitatively the interrelationships of carbon dioxide and oxygen on the flutter threshold.

The following experimental results indicate that in the diapausing *Cecropia* pupa, carbon dioxide affects spiracular behavior by acting peripherally on the spiracular mechanism and not on the central nervous system. The fourth abdominal spiracle did not respond when the fourth abdominal ganglion was perfused with pure carbon dioxide. Neither the experimental spiracle nor its controls opened fully and remained motionless when the *detracheated* fourth abdominal ganglion was perfused with gases in which the carbon dioxide concentration ranged from 7 to 30%. The same gas mixtures perfused systemically caused full and prolonged opening of all valves. Hence in the normal respiratory cycle, as intratracheal carbon dioxide increases, the spiracular mechanism responds long before tracheal carbon dioxide gets high enough to affect the central nervous system.

There is no evidence from these experiments that carbon dioxide concentrations below 30% affect spiracular behavior either directly or indirectly via the central nervous system. The possibility remains that a direct or indirect peripheral

response to carbon dioxide may be transmitted over sensory fibers to the central nervous system, which then alters the flow of information to the spiracular muscle. No specific receptors that may respond to changes in the concentration of carbon dioxide or oxygen have been found in the spiracular region although Beckel (1958) made an extensive histological search for such sensory elements. That the spiracular muscle can behave autonomously was shown by studies on the denervated muscle, which relaxed in high concentrations of carbon dioxide (Schneiderman, 1956; Beckel and Schneiderman, 1957); present results of partial denervation studies confirm their observations.

Hoyle (1960) concluded that in the locust spiracular muscle the partial pressure of carbon dioxide in the tracheae near the spiracle triggers spiracular opening. The present study indicates that this is also true for *Cecropia* pupae.

Van der Kloot (1963) passed 10% CO₂ + 90% O₂ over the isolated spiracular nerve-muscle preparation of a *Cecropia* pupa; he found that firing in the anterior lateral nerve ceased but some firing continued in the mid-lateral nerve. This indicates a central effect of carbon dioxide on nerves connected with the spiracular muscle. In contrast, our studies indicate that in the intact pupa carbon dioxide acts directly on the spiracular mechanism, not on the central nervous system. However, the possibility exists that sensory information concerning the carbon dioxide concentration or degree of stretch in the spiracular muscle is transmitted to the central nervous system, and this sensory input may affect motor output to the muscle.

Oxygen appears to act by a separate mechanism. In the present experiments the valve behavior of the fourth spiracle could be "dictated" by perfusing the detacheated ganglion with a gas of an appropriate concentration of oxygen. Hence oxygen appears to act via the central nervous system. The evidence for a peripheral response to oxygen is inconclusive but it appears that spiracular perfusions in which the oxygen concentration is 21% or more can mask a central response to oxygen. When the concentration of oxygen in the spiracular perfusion was above 21%, the fourth spiracle occasionally ceased fluttering even though the ganglionic oxygen concentrations used (5 to 10%) usually induced fluttering. This suggests some peripheral effect of oxygen at high concentrations. But in the normal physiological range of oxygen concentrations (5 to 21%) we have never observed a peripheral oxygen effect.

With regard to the nervous control of spiracular valve behavior, the results of the present studies are best evaluated by first considering the results of earlier studies (Beckel, 1958; Van der Kloot, 1963). Electrophysiological studies on the spiracular nerve and closer muscle (Van der Kloot, 1963) indicated that nerve AS which innervates the muscle (Beckel, 1958) is made up of at least four axons, of which two are from the mid-lateral nerve (Fig. 3). When the anterior lateral nerve was stimulated, the muscle twitched, and Van der Kloot concluded that in the intact pupa motor output from the anterior lateral nerve stimulates the closer muscle to contract. He also noted that when the carbon dioxide concentration was increased, efferent input through anterior lateral nerve ceased. This observation has important implications, for Beckel (1958) stated that the anterior lateral nerve is the median nerve which "arises in the ganglion preceding and courses between the ensheathed connectives to arise from the

ganglion succeeding" (page 91). When the mid-lateral nerve was stimulated, there was a delay before an action potential was observed in the anterior lateral nerve (Van der Kloot, 1963), and Van der Kloot concluded that fibers of the two synapse. Furthermore, nerve cord transection between the third thoracic and first abdominal ganglia eliminated the action potential in the anterior lateral nerve that resulted from stimulating the mid-lateral nerve. This result indicated that the synapse lay outside the ganglion of the stimulated mid-lateral nerve, and that the central nervous control of a spiracle lies in the ganglion of a different segment from that of the spiracle.

Van der Kloot found that the contracting spiracular muscle also receives impulses from the mid-lateral nerve, although the role of these axons is not known. In addition he found that even when motor output over the anterior lateral nerve to the muscle ceased (as it did when carbon dioxide was blown over the preparation), the muscle did not always relax since the muscle itself generated potentials. He drew no conclusions as to the exact roles of either nerve in the intact pupa but he suggested two possible functions for the spiracular nerve: The innervated muscle depends in part on excitatory output from the CNS to contract, and the CNS sends inhibitory impulses to the muscle when the carbon dioxide concentration increases.

The following results of the present experiments provide an alternative picture, and support the view that the ganglion and the spiracles which it primarily controls are located in the same segment and that the mid-lateral nerve plays a major role in triggering contraction of the spiracular muscle: (1) Variations in the local oxygen concentration to the fourth abdominal ganglion affect the fourth abdominal spiracle. (2) Detracheating the ganglion has the same effect as denervating the spiracle but does not affect adjacent spiracles. (3) Cutting the nerve cord between the third and fourth, and between the fourth and fifth abdominal ganglia does not change the responses of the fourth spiracle to spiracular or ganglionic perfusions of various gas mixtures. Nor does this operation affect the responses of the third and fifth spiracles although each was deprived of innervation from the fourth ganglion. (4) The behavior of the two spiracles in a segment is coordinated with each other but is not strictly synchronous with that of spiracles in other segments (Van der Kloot, 1963; Brockway and Schneiderman, 1967).

Cecropia pupae exhibit discontinuous respiration during which oxygen uptake is continuous whereas carbon dioxide release occurs periodically in "bursts." A carbon dioxide burst begins whenever the intratracheal concentration of carbon dioxide reaches the triggering threshold. The emphasis in this paper on the regulation of spiracular behavior by carbon dioxide and oxygen should not obscure the fact that the adaptive significance of discontinuous respiration, whether in insects or in other terrestrial arthropods (Robinson and Paim, 1969) is water conservation (Buck, 1958). What happens to the *Cecropia* pupa when the temperature in nature falls too low for the neuromuscular activity associated with discontinuous respiration to continue is discussed in a subsequent paper (Burkett and Schneiderman, 1974).

We thank Professor Peter Miller for his helpful comments on the typescript. This research was supported by a Biomedical Research Grant to the University of

Miami and AI-10527 to the University of California, Irvine from the National Institutes of Health.

SUMMARY

1. A technique was developed for perfusing the entire tracheal system of an insect with a known gas mixture at a rate that insured constant average intratracheal concentrations of oxygen and carbon dioxide. In addition to this technique, a method was devised to provide a ganglion with its own gas supply, separate from that of the rest of the insect. These techniques enabled us to show the following:

2. The behavior of the spiracles is unaffected when physiological concentrations of carbon dioxide are perfused through the ganglion. However, carbon dioxide perfused through the spiracles does affect valve behavior. Hence the primary response to carbon dioxide is peripheral.

3. The behavior of the spiracles is affected by varying the concentration of oxygen through the ganglion. Hence the primary response to oxygen is central.

4. Central nervous control of spiracular behavior resides primarily in the ganglion of the same segment in which spiracles are located.

5. Not only does oxygen affect the CO_2 -trigger threshold but also carbon dioxide affects the O_2 -flutter threshold. Moreover, the interaction between oxygen and carbon dioxide occurs peripherally.

LITERATURE CITED

- BECKEL, W. E., 1958. The morphology, histology and physiology of the spiracular regulatory apparatus of *Hyalophora cecropia* (L.) (Lepidoptera). *Proc. 10th Int. Congr. Entomol.*, 2: 87-115.
- BECKEL, W. E., AND H. A. SCHNEIDERMAN, 1957. Insect spiracle as an independent effector. *Science*, 126: 352-353.
- BROCKWAY, A. P., AND H. A. SCHNEIDERMAN, 1967. Strain-gauge transducer studies on intratracheal pressure and pupal length during discontinuous respiration in diapausing silkworm pupae. *J. Insect Physiol.*, 13: 1413-1451.
- BUCK, J. B., 1958. Cyclic CO_2 release in insects. IV. A theory of mechanism. *Biol. Bull.*, 114: 118-140.
- BUCK, J., AND M. KEISTER, 1955. Cyclic CO_2 release in diapausing *Agapema* pupae. *Biol. Bull.*, 109: 144-163.
- BURKETT, B. N., AND H. A. SCHNEIDERMAN, 1974. Discontinuous respiration in insects at low temperatures: intratracheal pressure changes and spiracular valve behavior. *Biol. Bull.*, 147: 294-310.
- EPHRUSSI, B., AND G. W. BEADLE, 1936. A technique of transplantation for *Drosophila*. *Amer. Natur.*, 52: 218-225.
- HOYLE, G., 1960. The action of carbon dioxide gas on an insect spiracular muscle. *J. Insect Physiol.*, 4: 63-79.
- LEVY, R. I., AND H. A. SCHNEIDERMAN, 1966a. Discontinuous respiration in insects—II. The direct measurement and significance of changes in tracheal gas composition during the respiratory cycle of silkworm pupae. *J. Insect Physiol.*, 12: 83-104.
- LEVY, R. I., AND H. A. SCHNEIDERMAN, 1966b. Discontinuous respiration in insects—III. The effect of temperature and ambient oxygen tension on the gaseous composition of the tracheal system of silkworm pupae. *J. Insect Physiol.*, 12: 105-121.
- MILLER, P. L., 1966. The regulation of breathing in insects. Pages 279-354 in J. W. L. Beament, J. E. Treherne, and V. B. Wigglesworth, Eds., *Advances in Insect Physiology*, Vol. 3. Academic Press, New York.

- ROBINSON, G. L., AND U. PAIM, 1969. Regulation of external respiration by the book-lung spiracles of the spiders, *Araneus diadematus* Clerck and *A. marmoreus* Clerck. *Can. J. Zool.*, **47**: 355-364.
- SCHNEIDERMAN, H. A., 1956. Spiracular control of discontinuous respiration in insects. *Nature*, **117**: 1169-1171.
- SCHNEIDERMAN, H. A., 1960. Discontinuous respiration in insects: role of the spiracles. *Biol. Bull.*, **119**: 494-528.
- SCHNEIDERMAN, H. A., AND A. N. SCHECHTER, 1966. Discontinuous respiration in insects. V. Pressure and volume changes in the tracheal system of silkworm pupae. *J. Insect Physiol.*, **12**: 1143-1170.
- SCHNEIDERMAN, H. A., AND C. M. WILLIAMS, 1955. An experimental analysis of the discontinuous respiration of the *Cecropia* silkworm. *Biol. Bull.*, **109**: 123-143.
- VAN DER KLOOT, W. G., 1963. The electrophysiology and the nervous control of the spiracular muscle of pupae of the giant silkmths. *Comp. Biochem. Physiol.*, **9**: 317-333.
- WILLIAMS, C. M., 1946. Physiology of insect diapause: the role of the brain in the production and termination of pupal dormancy in the giant silkworm *Platysamia cecropia*. *Biol. Bull.*, **90**: 234-243.